CHLORAMPHENICOL

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I. Introduction

The discovery of chloramphenicol[2] was first announced by Ehrlich *et al.* (36) and independently by a group at the University of Illinois (17). Preliminary chemical properties were described by Bartz (6), and a complete structural determination was reported by Rebstock *et al.* (84). The structure is shown in figure 1.

This antibiotic is unique in that it was the first natural product found that contained a nitro group and also the first natural product which was a derivative of dichloroacetic acid. Several generic names have been used for chloramphenicol. The preferred Chemical Abstracts name is D-*threo*-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl]-acetamide, but the name more commonly seen in the literature is D(−) *threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1,3-propanediol (84). It can be seen that there are two asymmetric carbon atoms, leading to four possible stereoisomers. All four isomers have been synthesized (26), and the two *erythro* isomers are biologically inactive, whereas the L(+) *threo* isomer has less than 0.5 per cent of the activity of the natural D(−) *threo* isomer.

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[2] The trade name of Parke, Davis and Company for chloramphenicol is Chloromycetin.

Chloramphenicol was the first of the clinically useful antibiotics to be synthesized and the only one which is marketed in synthetic form today. Because of its relatively simple structure, a large number of modifications of this antibiotic have been prepared and tested. A number of biochemists and physiologists have viewed its simple structure and have been encouraged to work on it, possibly with the feeling that what looks simple must have a simple mode of action. This view has proved to be erroneous, but has led to a large number of interesting studies. In recent years chloramphenicol has become a tool of molecular biologists and geneticists working on the synthesis and function of nucleic acids, since it can inhibit protein synthesis while allowing continued nucleic acid synthesis.

The work on this antibiotic is found in a wide variety of journals, and there has been no review of most of this literature. Early work on mode of action was reviewed briefly by Smith (88), and a review of structure-activity studies has been published by Hahn *et al.* (52). Woodward and Wisseman (100) have reviewed extensively the clinical uses of chloramphenicol. But the present review is the first to attempt any detailed coverage of most aspects of this drug. I have almost 1000 references dealing wholly or in part with chloramphenicol, and these exclude all clinical papers. Obviously this review cannot discuss

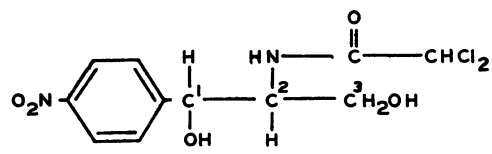

Figure 1. Structure of chloramphenicol

each paper. I have attempted to examine these references critically, and to eliminate any which seemed fragmentary, inconclusive, or erroneous. This selection has still left a large number to consider. In certain fields, in which a large number of similar papers have been published, only the first or the key reference is discussed. Wherever possible, citations to papers which review portions of the field are given. To limit the review further, only certain areas are treated in detail. Clinical work has been completely ignored, and most work on such aspects as antibiotic combinations, resistance, cross resistance, and host-parasite relations has been ignored.

II. Relationship of Structure to Antibacterial Activity

Because of the great clinical utility of chloramphenicol and its relative ease of chemical synthesis, it was natural that a large number of chloramphenicol analogues would be made and tested for antibacterial activity. Although no analogue has proved superior to the natural antibiotic, the results of these studies have revealed some of the structural requirements for antibacterial activity in the chloramphenicol series.

The relative activities of the four isomers have been compared by Maxwell and Nickel (76); only the D(−) *threo* isomer showed any significant activity. However, Hahn *et al.* (54) have shown that the synthesis of a D-glutamic acid polypeptide by *Bacillus subtilis* is not inhibited by the D(−) *threo* isomer, but is inhibited by the L(+) *erythro* isomer. Since both these isomers have the OH group on carbon 1 in the same position, it is presumed that the stereochemical configuration at carbon 1 is essential for any biological activity, whereas the configuration at carbon 2 determines whether L-polypeptide (protein) or D-polypeptide synthesis will be inhibited.

The structure of the propanediol moiety is critical for activity. If either hydroxyl is replaced by hydrogen atoms, all activity is lost, and the same is true if the hydroxyl groups are esterified. The propane chain cannot be extended, as in 1,3-diol-3-dimethylpropane or in butane-1,3-diol, without loss of activity (52). In addition, substitution of a methyl group for the hydrogen atom on carbon 2 leads to a loss of activity (25).

In the acetamide side chain, the size of the constituent and its electronegativity influence the activity, but there is no absolute requirement for the chlorine atoms (52). Also, the free base of chloramphenicol, resulting from the complete removal of the dichloroacetamide side chain, has 1.8 per cent of the activity of the parent drug (84). Further, if the free hydrogen on the nitrogen atom is replaced with a methyl group, all activity is lost (25).

The aryl nitro group is not essential for activity, since a number of analogues with different substituents on the aromatic ring retain partial or total activity (52). The geometry of the aryl group is also of little importance, as long as this portion of the molecule is conjugated with the side chain (86). Shemyakin *et al.* (86) postulate that the most important feature of this grouping is its polarizing ability, the geometry of the radical carrying it having little effect.

Thus, certain features of the chloramphenicol molecule seem important for its antibacterial activity. It is necessary to realize that a molecule is a three-dimensional entity and is poorly represented by two-dimensional models on paper. Although there is no evidence on the configuration that this antibiotic assumes in solution, it is possible that it adopts a curled configuration, with hydrogen bonds occurring between the hydroxyls of carbons 1 and 3, forming a six-membered ring including carbon 2. The hydrogen atom on the nitrogen is considered to be free. Such a configuration would be especially suitable for interaction with the polar groups of a protein chain, since the two hydroxyls and the nitrogen atom of the peptide link would all be pointing outward from the molecule (35). The propane side chain, and the hydrogen atoms on carbon 2 and 3 and the amide nitrogen are assumed to be the points of attachment with an enzyme (25). This portion is considered to be the *specific* pharmacodynamic portion of the molecule. This specific portion is then modified in its chemical and electronic properties by the less specific *p*-nitrophenyl and dichloroacetyl portions, and these parts are probably not embedded directly in the enzyme

protein matrix, but held upon the surface. Collins *et al.* (25) consider the *p*-nitrophenyl moiety to serve in the manner of a grappling hook to hold the antibiotic to the surface of the enzyme, whereas Shemyakin *et al.* (86) consider the electronic behavior of this moiety to be the most important aspect, perhaps modifying the propanediol portion of the molecule in such a way as to elicit a pharmacological effect without in itself being involved in the attachment of the antibiotic to the enzyme. Any further speculation along these lines will not be fruitful until more about the site of action of chloramphenicol is known.

III. Spectrum of Organisms Inhibited

Chloramphenicol is effective in inhibiting a wide variety of bacteria, from practically all families, at concentrations between 1 and 10 μg per ml (77, 96). This is in contrast to penicillin, which inhibits all bacteria, but at widely varying concentrations, so that some species may be inhibited by 0.001 μg per ml, whereas others require 1000 μg per ml. Very few bacterial species are completely inhibited by concentrations of chloramphenicol less than 1 μg per ml.

The spirochetes and the filamentous bacteria are also inhibited by chloramphenicol. Some groups such as the myxobacteria have apparently never been tested. There have been conflicting reports on the sensitivity of the pleuropneumonia-like organisms (genus *Mycoplasma*), since some workers have found them resistant, whereas others have found them to be sensitive. The "killer" particle in *Paramecium aurelia*, which may be a small obligately parasitic bacterium, is selectively inhibited by chloramphenicol (15, 97).

Viruses and rickettsias which are affected by chloramphenicol include: lymphogranuloma, psittacosis, epidemic typhus, murine typhus, scrub typhus, rickettsialpox, Rocky Mountain spotted fever, and Q fever (77). Those viruses which are unaffected by this antibiotic include: vaccinia, variola, St. Louis encephalitis, Japanese encephalitis, rabies, polio, Theiler's intestinal virus, mumps, influenza, distemper, Newcastle disease, chick bronchitis, and laryngotracheitis (77). The bacterial viruses are apparently not affected directly, but their growth is inhibited indirectly through effects on the metabolism of the host (12).

A number of fungi and yeasts were completely resistant to 1000 μg per ml (77). Most protozoa are also very resistant (77), although *Tetrahymena pyriformis* was inhibited by 40 μg per ml (51). Green algae were resistant, whereas certain blue-green algae were inhibited (41). A number of primary explants of animal cells have also been found to be very resistant (43, 80), although it has recently been reported that an established human cell line is completely inhibited by 20 μg per ml (32). No reports have been found on the inhibition of growth of whole animals by chloramphenicol, although the results of observations of blood dyscrasias in humans (89) might be an indication of inhibition of growth of the hematopoietic cells.

From this brief survey it is seen that chloramphenicol is primarily a bacteriostatic agent, inhibiting all the true bacteria and organisms considered quite closely related (rickettsias, blue-green algae, spirochetes) at low concentrations. Certain protozoa and animal cell lines also seem to be inhibited, whereas fungi and plants are quite resistant. This selectivity is the basis of usefulness of chloramphenicol as a chemotherapeutic agent.

IV. Resistance of Bacteria to Chloramphenicol

As discussed above, all species of bacteria seem to be sensitive to chloramphenicol and are inhibited completely by concentrations from 1 to 10 μg per ml. A large number of papers have reported the development of resistance to chloramphenicol which, aside from its clinical aspects, is of genetic and biochemical interest.

An extensive analysis of the genetic basis of resistance to chloramphenicol has been performed by Cavalli and Maccacaro (19, 20) and Cavalli (18). These workers used strains of *Escherichia coli* K-12 in which genetic crosses could be performed by mating. The strains used had several growth factor requirements as well as fermentation, phage resistance, and drug resistance markers. These strains were originally sensitive to 5 to 10 μg per ml of chloramphenicol. By plating large populations of cells on agar containing 20 to 49 μg per ml of the antibiotic, one-step resistant mutants could be isolated. It was possible to show the mutational origin of these strains by use of fluctuation test experiments (18), although detailed quantitative studies of the mutation rate could not be deter-

mined because of technical difficulties. Most of these one-step resistant mutants could be mapped in the region between methionineless and bacteriophage T 6 resistance, although mutants at other loci were also found. A number of independent one-step mutants were isolated and crossed to another resistant strain to determine allelism and interaction between mutants. At least four or five different loci were found, all conferring a similar degree of resistance. Furthermore, crosses of two resistant strains yielded a significant percentage of fully sensitive recombinants as well as recombinants with a higher resistance than either parent.

These workers also isolated mutants resistant to high levels of chloramphenicol (up to 1000 μg per ml) by periodic transfer to higher levels of drug. This development of high level resistance was always stepwise. These resistant strains were then crossed to sensitive ones and recombinants selected on the basis of the nutritional markers. When these recombinants were then tested for resistance, all levels of resistance were observed, including some fully sensitive strains. If two highly resistant strains were crossed, fully sensitive recombinants could be found. These authors interpret these results to indicate that there are a number of genes at different loci which confer low levels of chloramphenicol resistance, and that these loci can interact either in a positive way, leading to higher resistance, or in a negative way, leading to lower resistance or even sensitivity. It is possible that repeated selection in isolating high level resistant strains tends to build up a polygenic system with many positive interactions, and recombination is likely to break down such positively interacting systems and may reveal negative interactions by combining in one genome, loci which do not interact positively.

Strains resistant to chloramphenicol have been tested for cross resistance with a number of other antibiotics. A common experience of most workers is that enteric bacteria resistant to chloramphenicol are cross resistant with the tetracyclines, whereas species of other families do not show such cross resistance (91–94). A preliminary genetic analysis of the cross resistance between chloramphenicol and oxytetracycline (Terramycin) was performed by Cavalli (18). He found that high level resistance to oxytetracycline conferred high level resistance to chloramphenicol, whereas high level resistance to chloramphenicol conferred only low level resistance to oxytetracycline. In crosses between oxytetracycline-resistant strains and sensitive strains, it was shown that recombinants which showed oxytetracycline resistance were always chloramphenicol-resistant, whereas recombinants which were sensitive to oxytetracycline were always chloramphenicol-sensitive. In mapping studies, he showed that a group of genes located to the left of the methionineless region conferred resistance to both chloramphenicol and oxytetracycline, whereas another group of genes located in the methionineless, threonineless, leucineless (M-TL) region had little effect on oxytetracycline resistance but conferred chloramphenicol resistance.

The lack of cross resistance between chloramphenicol and the tetracyclines in species other than the enteric bacteria presumably reflects diverse genetic backgrounds and differing genetic mechanisms controlling resistance in different organisms. Now that genetic recombination techniques are more widely available, it would be interesting to study the genetics of chloramphenicol resistance in other organisms.

Statements have often been made in the literature that antibiotics which show cross resistance should have similar modes of action. The data cited here show the fallaciousness of this reasoning, since one would have to conclude that chloramphenicol and the tetracyclines have similar modes of action in *E. coli*, but different modes of action in *Staphylococcus aureus*. In reality, occurrence of cross resistance may merely serve to indicate that in a particular organism, the genetic loci for resistance are the same, as shown by Cavalli (18).

A number of workers have compared the resistant strains that they isolated with the parent strain for differences in antigenic characteristics, diagnostic biochemical characteristics, or other physiological properties. The most common report has been a loss or reduction in the H antigen in chloramphenicol-resistant enteric bacteria. Other changes reported have been increased or decreased growth factor requirements, decreased growth rate, changes in respiratory activity, or changes in sensitivity to other inhibitors. The mutants with high level resistance that Cavalli and Maccacaro (20) isolated had slower growth rates and tended to give mucoid colonies. However, in recombination experiments, both these characteristics were separated

from chloramphenicol resistance, so that slowly growing sensitives or mucoid sensitives were isolated as well as rare nonmucoid resistants. These results are best explained in terms of the polygenic hypothesis (see above), in that some of the loci involved in resistance are modifiers of other loci and do not in themselves confer any resistance. These modifier loci may then confer other characteristics on the organism merely incidental to their effects on chloramphenicol resistance.

The biochemical basis of chloramphenicol resistance is unknown. It is clear that resistance is not due to increased production of enzymes that destroy the antibiotic, since most of these enzymes are not produced in greater amounts in resistant than in sensitive cells (20, 78). Loss of permeability to the antibiotic is another possibility, but this has not been explored, probably because of the unavailability of radioactive chloramphenicol. Ramsey (83) felt that he had shown that chloramphenicol resistance in *S. aureus* was due to a modification of the active site of the antibiotic, but his data are not convincing.

V. Combined Action with Other Antibiotics

Because of clinical interest, there have been a large number of studies on the combined action of chloramphenicol and other antibiotics. However, this type of study is more complicated than is often realized, and results obtained are not always subject to clear interpretations. This problem is discussed in detail by Loewe (71) for pharmacological agents in general, and briefly by Ciak and Hahn (22) in relation to antibiotic combinations. Fortunately, many of the data for chloramphenicol seem to be amenable to straightforward interpretations, so that it will not be necessary in this review to consider the theoretical problems. To simplify matters, only data from short-term experiments (4 hours or less) will be considered, since in long-term experiments there are many secondary actions which might arise and complicate the results.

As noted in another section, chloramphenicol is primarily a bacteriostatic agent and has little or no killing action on growing or resting cells in short incubation periods. It is an effective inhibitor of growth and consequently might be expected to antagonize antibiotics which are able to act only on growing cells.

Jawetz *et al.* (65) first reported the antagonism of the killing action of penicillin by chloramphenicol. Kirby and Burnell (68) showed that the lysis induced by penicillin in *S. aureus* was delayed considerably by chloramphenicol, and Prestidge and Pardee (81) showed that this was true with *E. coli* as well. Since the recent work on the mode of action of penicillin showing that it can bring about dealth only in cells able to grow, these results are easily understood. One important point in these studies is that the chloramphenicol must be added at the same time or before the penicillin and that it has pregressively less effect the later it is added after the penicillin.

Jawetz *et al.* (64) also showed that streptomycin was antagonized by chloramphenicol. Recent work (2, 3) has shown that streptomycin is bactericidal only to growing cells, although the situation is complicated by the binding of streptomycin by the cells. Streptomycin is passively bound to the exterior of the cell in small amounts, and this initial binding is not inhibited by chloramphenicol. If the cells are able to grow, the passively bound streptomycin brings about damage to the cell membrane, leading to an increased permeability of the cell to various small molecules, including streptomycin itself, and a secondary uptake of streptomycin occurs. Chloramphenicol prevents this cell membrane damage from occurring, so that the increased permeability, which is responsible for the bactericidal effects of streptomycin, does not occur. Its antagonistic action is thus due to the fact that it inhibits the growth which is necessary for the membrane damage to occur. Because of this, it antagonizes both the killing effect and the secondary uptake of streptomycin but not the primary uptake.

It should be emphasized that the antagonism of penicillin and streptomycin action is not unique to chloramphenicol. A similar antagonism occurs if growth is inhibited in any one of a number of nonlethal ways, such as by the use of amino acid analogues, the withholding of essential growth factors in auxotrophs, incubation at 0 C, incubation in the absence of carbon, nitrogen, or energy sources, or the use of other bacteriostatic antibiotics.

A careful study was made of the interaction of chloramphenicol and the tetracyclines by Ciak and Hahn (22). These antibiotics show exactly additive responses with no evidence of

either antagonism or synergism. Since both chloramphenicol and the tetracyclines inhibit protein synthesis, it is thought by these workers that these two groups of antibiotics are additive because they block concurrent metabolic pathways which contribute to protein synthesis. In addition, erythromycin and chloramphenicol are additive and also show similar modes of action (14).

Although there have been reports of synergistic action of chloramphenicol with antibiotics, these reports have little meaning because of the difficulties of defining synergism (71). In conclusion, it can be stated that chloramphenicol is additive with those antibiotics which have similar modes of action (erythromycin, the tetracyclines) and antagonistic with those antibiotics which are bactericidal to growing cells (penicillin, streptomycin). Further, these results do not seem to depend on the species of bacteria used as test organism, in contrast to the high species specificity of the cross resistance studies (see above).

VI. Action on Bacterial Growth, Viability, and Morphology

Before biochemical observations related to antibiotic action have any certain meaning, it is essential to know the general effects that an agent has on bacterial growth, viability, and morphology. A careful study of the bactericidal and bacteriostatic effects of chloramphenicol has been made by Fassin *et al.* (38), with the use of the millipore filter to remove unbound chloramphenicol from the cells. They found chloramphenicol to be strictly bacteriostatic to the following organisms: *Salmonella typhosa*, *Salmonella schottmuelleri*, *Brucella* spp., enterococcus, *Escherichia coli*, viridans streptococcus, *Streptococcus pyogenes*, and *Staphylococcus aureus*. Those organisms to which chloramphenicol was bactericidal were: 12 nonpathogenic, gram-positive spore formers (presumably *Bacillus* species) and *Shigella flexneri*.

In detailed studies with a *Salmonella typhosa* strain, these workers found that progressively higher concentrations of chloramphenicol resulted in progressively more inhibition of growth, until complete inhibition was achieved at 7.5 μg per ml of chloramphenicol. Increases in concentration to as high as 1500 μg per ml did not result in any killing, at least during the incubation period used. These results show clearly the strictly bacteriostatic action of chloramphenicol on this strain. With longer periods of incubation, various workers have noticed bactericidal effects, but the killing observed was apparently a secondary effect and not due to a primary action of chloramphenicol.

Optical density readings to measure the inhibition of growth of logarithmically growing cultures of *E. coli* have been made (22, 61). A progressive decrease in growth rate was found as the concentration of chloramphenicol was increased, until complete inhibition was achieved at 10 μg per ml. These results correspond quite closely with the viable count measurements of Fassin *et al.* (38), but optical density readings do not measure any killing action of an antibiotic. In contrast to chloramphenicol, when even very high concentrations of penicillin were added to logarithmically growing cultures, optical density still continued to increase for a short time before lysis began and a sharp drop occurred (22).

Further studies were made by Fassin *et al.* (38) to determine the effects of various factors on chloramphenicol bacteriostasis. Addition of the antibiotic at different stages of growth or at different cell densities produced substantially the same results. In a study of the relationship between chloramphenicol action and growth rate it was possible to conclude that the rate of growth as controlled by incubation temperature does not influence the response to chloramphenicol at temperatures below 37 C. However, at temperatures above the optimal growth temperature, the cells were more sensitive to chloramphenicol inhibition.

When a gram-positive aerobic spore former was used, low concentrations were bacteriostatic, whereas higher concentrations were bactericidal without any lag. This bactericidal action was markedly reduced when the growth rate was lowered and did not occur at all with cells incubated in a medium which would not support growth. These experiments were done at cell densities too low to detect lysis, but Bernlohr and Novelli (7) have reported that high concentrations of chloramphenicol bring about lysis in *Bacillus licheniformis*. It seems, therefore, that chloramphenicol is bacteriostatic to most species but bactericidal to bacilli. It is interesting that the killing action of chloramphenicol against the gram-positive spore formers began without any lag and did not occur in nongrowing cells. Because

of possible differences in growth processes and structural organization in these bacilli, further studies of this chloramphenicol death might be worth undertaking. Because there have been so few detailed studies of bacteriostatic and bactericidal effects of chloramphenicol, workers should be cautioned to determine the killing action of chloramphenicol on the particular organism with which they are working before assuming that it does not occur.

A few observations have been made on morphological changes taking place in bacteria growing in the presence of chloramphenicol. Because of the uncertainties connected with cytological studies on bacteria, the meaning of these observations is not clear, but they should be considered along with other effects of chloramphenicol on bacteria.

Cells growing in the presence of concentrations of chloramphenicol too low to cause complete inhibition of growth have exhibited abnormal shapes (82). Although these abnormal shapes have been called L forms by some workers, they do not continue to divide and grow and are apparently not analogous to the L forms induced by penicillin (31).

Changes in the nuclear bodies have also been observed. Bergerson (8), using acid-Giemsa staining in *E. coli*, first reported that the nuclear material became arranged in long, irregular bars at concentrations of chloramphenicol (2.5 μg per ml) not sufficient to cause complete inhibition of growth. Hahn *et al.* (53) found progressive increases in the size of the bacterial nuclei upon continued incubation with 10 μg per ml of chloramphenicol and a return to normal when the bacteria were removed from the antibiotic and placed in fresh medium. These workers correlated the increase in nuclear size with an increase in deoxyribonucleic acid (DNA) content of the cells and presumed that the nuclear changes were a reflection of the changes in DNA. Very similar nuclear changes were observed in *Bacillus megaterium* by DeLamater *et al.* (29), although these changes were not specific for chloramphenicol but were also observed with the tetracyclines, erythromycin, carbomycin, and streptomycin. An electron microscopic study (67) of ultra-thin sections of chloramphenicol-treated cells showed changes similar to those observed in the light microscope.

VII. Reversal by Metabolites and Other Chemical Compounds

The apparent simplicity of the structural formula of chloramphenicol has tempted a number of people to assume that it must be an analogue of some known growth factor. This has led to a number of experiments in which various test organisms were cultured in synthetic media and the inhibitory power of chloramphenicol determined with and without added supplements.

When very low concentrations of chloramphenicol are used (ca. 1μg per ml), some reversal of its action can be detected with the use of various additives (39, 40, 87, 101). When even slightly higher concentrations of the antibiotic are used (ca. 2 μg per ml), no reversal is found. Most of the reversing agents are aromatic compounds. Smith (87) has shown reversal by a number of aromatic compounds which are not normal metabolites (i.e., 2, 4-dinitrophenol). Although Woolley (101) postulated that chloramphenicol was a phenylalanine analogue, the large number of aromatic compounds which effect minimal reversal indicates some nonspecific reversal mechanism. Since recent work (23) has shown the presence of specific transport systems for the uptake of various compounds, it is possible that chloramphenicol enters by an aromatic transport system which serves to concentrate it in the cell when it is present externally in small amounts. This transport system might then be antagonized by other aromatic compounds. However, when the concentration of chloramphenicol is raised slightly higher, bacteriostatic concentrations in the cell might then be achieved by nonspecific passive diffusion, which would not be antagonized by aromatic compounds. Such reasoning might explain why workers like Woolley (101) and Foster and Pittillo (40) could produce reversal of inhibition of 1 μg per ml of chloramphenicol but not of 2 μg per ml. Conclusions of this sort might be established if the uptake of chloramphenicol into the cell could be studied.

VIII. Inhibition of Physiological Processes

A. Action on Energy-Yielding Processes

Gale and Paine (45) and Gale and Folkes (44) found no effect of very high concentrations of chloramphenicol on fermentation or respiration

of glucose. Hahn *et al.* (55) found no inhibition of bioluminescence or motility, both processes requiring energy. Also, phosphorylation in glucose dissimilation was unaffected.

In view of these results, the observations of Kushner (69) that chloramphenicol strongly inhibits the oxidation of succinate, fumarate, malate, and α-ketoglutarate in *Pseudomonas fluorescens* may seem anomalous until it is realized that Kushner grew his cells in yeast extract-peptone medium and tested them for oxidation without adapting them to the desired substrate. Chloramphenicol was indeed inhibiting the oxidation of these substrates but probably was doing so through inhibition of the induced synthesis of the necessary enzymes, and not through any primary action on oxidative reactions. This interpretation is supported by the fact that the inhibitions were less if the antibiotic was added some time after the substrate. These considerations should serve as a warning of the intricacies involved in interpreting data and show the importance of using a well designed experiment for studying the mode of action of antibiotics.

Because chloramphenicol does not inhibit respiration, it is a useful inhibitor for many types of studies involving protein synthesis. Traditional respiratory inhibitors, such as cyanide, azide, and dinitrophenol, prevent all cellular functions from proceeding.

B. Action on Permeation Processes

Gale and Paine (45) showed that chloramphenicol did not inhibit the accumulation of free glutamic acid in the cell, whereas it strongly inhibited the conversion of glutamic acid to a combined form. Other workers have also shown that there is no inhibition of accumulation of free amino acids (24, 56). Since amino acid uptake is not inhibited, while the incorporation of these amino acids into protein is inhibited, there is a rapid increase in the size of the free amino acid pool after addition of chloramphenicol, and the rate of accumulation in the pool becomes quite close to the rate at which these amino acids had been incorporated into protein (56).

There is also no inhibition of the uptake of β-galactosides (23). Presumably all permeation processes will function normally in the presence of chloramphenicol, although there have apparently been no studies on the uptake of ions.

C. Action on Synthesis of Small Molecules

There is no direct evidence that chloramphenicol inhibits the synthesis of any of the small molecules that serve as building blocks for the cell, and there is much indirect evidence that it is without effect on these syntheses. Mandelstam (73) has shown that in cultures of *Escherichia coli* growing in glucose-salts medium, so that all essential amino acids must be synthesized, there is a marked increase in the amount of all free amino acids upon addition of chloramphenicol, presumably because synthesis of these continues while incorporation into protein is blocked. This leads to an increase in amino acid concentration within the cellular pool and also an excretion of amino acids into the medium. Since the antibiotic does not inhibit nucleic acid synthesis (see below), it would be expected that the synthesis of purine and pyrimidine bases, the ribonucleotides, and deoxyribonucleotides would not be inhibited. Direct evidence for this is provided in the work of Wisseman *et al.* (98), in which incorporation of C^{14}-glycine into nucleic acid adenine and guanine was unaffected by concentrations of chloramphenicol that markedly reduced the glycine incorporation into protein. The incorporation of inorganic P^{32} into nucleotides is also not inhibited (5).

There have been no studies of effects of chloramphenicol on fatty acid synthesis, probably because methodology in this field is not so advanced as in the other areas.

We may tentatively conclude that chloramphenicol does not inhibit growth by preventing the synthesis of some small molecule required as a building block. This inference is further supported by the fact that chloramphenicol is a good inhibitor of the growth of the lactic acid bacteria which cannot synthesize their own amino acids, purines, and pyrimidines, but must have them supplied externally.

D. Inhibition of Synthesis of Large Molecules from Small Molecules

1. Inhibition of protein synthesis. When chloramphenicol is added to growing cells in concentrations of 10 μg per ml and above, protein synthesis, as measured in a wide variety of ways, is inhibited 95 to 100 per cent. This was first shown by Gale and Folkes (44) for *Staphylococcus aureus* and by Wisseman *et al.* (98) for *E. coli* and has been confirmed by many workers

in a wide variety of organisms. Since total protein synthesis is inhibited, it would be expected that the synthesis of individual enzymes or other specific proteins would also be inhibited.

A survey of the literature reveals inhibition of synthesis of the following specific proteins by chloramphenicol: aldolase, alkaline phosphatase, amylase, δ-aminolevulinic acid dehydrase, δ-aminolevulinic acid synthetase, carbamyl phosphate synthetase, catalase, flagella, β-galactosidase, β-galactoside permease, lytic enzyme of *Bacillus subtilis*, maltose permease, nitrate reductase, ornithine transcarbamylase, phosphomonoesterase, phage protein, protease from mouse pancreas, ribonuclease of *E. coli*, streptolysin S, succinic dehydrogenase, tetrathionate reductase, and tryptophan synthetase. In all these cases it was shown that chloramphenicol did not inhibit the activity of the protein but only its synthesis.

In this list are several proteins synthesized in animal or plant systems. It usually took considerably greater concentrations of chloramphenicol to inhibit these syntheses, but this is in line with the higher concentrations of chloramphenicol required to inhibit growth in these organisms. Note also that some of the systems were considered cell-free, but the controversial nature of these does not detract from the fact that synthesis was inhibited.

The wide variety of enzyme syntheses inhibited indicates the generality of chloramphenicol action. In this regard it has become common for workers to use chloramphenicol inhibition as evidence for or against a synthetic process, as opposed to a precursor-protein conversion, in studying protein synthesis. For instance, studying toxin formation in *Clostridium botulinum*, Bonventre and Kempe (10) showed that chloramphenicol did not inhibit the appearance of toxin activity, even though it effectively inhibited total protein synthesis, and they interpreted this observation as evidence of a release or activation of a previously synthesized protein, rather than of synthesis *de novo*. In a study of apparent cell-free synthesis of β-galactosidase, Kameyama and Novelli (66) showed inhibition by chloramphenicol and used this observation as evidence for actual enzyme synthesis and not for release. In view of the massive number of observations on inhibition of protein synthesis by chloramphenicol, such conclusions seem justified.

Although this antibiotic strongly inhibits protein synthesis, it has little or no effect on protein degradation in nongrowing *E. coli* (74); the amino acids which are released from protein accumulate, since their reincorporation into protein is inhibited.

Since the pioneering work of Gale and Paine (45), a large number of observations have been made on chloramphenicol inhibition of incorporation of labeled amino acids into material insoluble in hot trichloracetic acid. As mentioned previously, chloramphenicol does not prevent the accumulation of free amino acids in the cell, so inhibition of incorporation into stable linkages indicates an inhibition of some later stage in the incorporation process. Various workers have reported the inhibition of incorporation of various C^{14}-labeled amino acids and other labeled protein precursors. Inhibition of incorporation is found in animal, plant, and microbial systems, in both whole cells and in cell-free preparations.

Studies using C^{14} amino acids may be complicated by the fact that these amino acids may be converted into nonamino acid molecules (e. g., glycine to purines), or the amino acids may be incorporated into some nonprotein linkage stable in hot trichloracetic acid, such as the cell wall (see below).

2. Inhibition of peptide synthesis. Although in most organisms, amino acids combined in peptide linkage are usually in the L form, there are in bacteria some peptides containing D-amino acids. In a very interesting paper, Hahn *et al.* (54) showed that the synthesis by *Bacillus subtilis* of a polypeptide which was composed exclusively of D-glutamic acid was not inhibited by chloramphenicol (the D (−) *threo* isomer), although it was quite effectively inhibited by the L (+) *erythro* isomer. This observation seems to indicate some specific involvement of chloramphenicol in peptide bond synthesis in which the antibiotic is acting at the site where the amino acids are joined together. Unfortunately this observation has not been pursued further.

Chloramphenicol does not inhibit the incorporation of amino acids into the cell wall mucopeptide of *S. aureus* (75). However, several of the amino acids in this mucopeptide are in the D configuration, so it is not clear whether this lack of inhibition by chloramphenicol is due to a fundamentally different process in cell wall synthesis or whether the wrong isomer of the antibiotic has been used. There have apparently

been no attempts to inhibit cell wall synthesis with the L (+) *erythro* isomer.

The synthesis of bacitracin, a peptide containing both L- and D-amino acids, is not inhibited by chloramphenicol as shown by Bernlohr and Novelli (7), although these workers have shown that bacitracin is released during sporulation and may be a fragment of the cell wall that had been synthesized earlier.

The synthesis of the simple tripeptide glutathione is not inhibited by chloramphenicol (85).

3. Nitrogen fixation. An observation of some interest (16) is that chloramphenicol does not inhibit nitrogen fixation in *Azotobacter* but does inhibit protein synthesis, so that the acid-soluble intermediate products of nitrogen fixation accumulate. The antibiotic may thus be useful as a tool in analyzing the first steps in nitrogen fixation.

4. Inhibition of intermediate steps in protein synthesis. a. Amino acid activation. This process is not inhibited even by high concentrations of chloramphenicol. This was first reported by Demoss and Novelli (30) with bacterial systems and has been confirmed by a variety of workers in both bacterial and mammalian systems.

b. Transfer of activated amino acids to soluble ribonucleic acid (RNA). Lacks and Gros (70) have shown in growing cells of *E. coli* that externally supplied labeled amino acid can become attached to soluble RNA. When chloramphenicol is present, the amount of amino acid attached to soluble RNA increases, whereas the incorporation of amino acid into protein is completely inhibited. This is good evidence that chloramphenicol does not inhibit transfer of activated amino acids to soluble RNA. Other workers have shown that amino acid attachment to soluble RNA is not inhibited in cell-free systems (bacterial systems (90), animal systems (59, 72)).

c. Transfer of amino acid from soluble RNA to protein. This process may involve a number of steps, all unknown, which can be grouped in two classes: polymerization of amino acids and coding of amino acids. Since chloramphenicol brings about the accumulation of amino acid-soluble RNA complexes, it follows that it inhibits the transfer of amino acids from soluble RNA to protein. The exact site of action cannot be determined until this process has been dissected further. It seems reasonable to infer that the antibiotic does not inhibit the coding steps but does prevent the polymerization steps leading to the synthesis of peptide bonds.

Recently, Hunter and Goodsall (63) have reported some preliminary observations on the incorporation of a labeled amino acid into a phospholipid fraction, from which it was subsequently transferred to protein. Chloramphenicol inhibited the incorporation of the amino acid into the phospholipid. It is not known how this observation relates to other studies on protein synthesis, but it is possible that the amino acid is transferred to the phospholipid from soluble RNA. This then might be the step that is inhibited by chloramphenicol. Further work in this area will be awaited with interest.

5. Inhibition of protein synthesis by other antibiotics. Other antibiotics whose actions are similar to that of chloramphenicol include the tetracyclines, erythromycin (and probably other macrolide antibiotics such as oleandomycin and spiramycin) and puromycin. Recently Yarmolinsky and de la Haba (102) have shown that puromycin does not inhibit amino acid activation and transfer to soluble RNA but does inhibit transfer from soluble RNA to protein. It seems to have an action identical to that of chloramphenicol, but unfortunately there has been little work with it in bacteria. By inference, the other antibiotics listed above may also act between soluble RNA and protein. As the details of these steps become clear, it will probably be possible to localize the site of action of each antibiotic. Because of their wide structural differences, it would be doubtful that they all act at exactly the same place.

6. Effects on nucleic acid synthesis. When nucleic acid synthesis is measured by a variety of methods, it is found that chloramphenicol has little effect on this process. Gale and Folkes (44) first reported a stimulation of nucleic acid synthesis in *S. aureus*, and various workers have reported either a slight stimulation or no significant inhibition in *E. coli* (98).

Both RNA and DNA can be synthesized in the presence of chloramphenicol in growing cells. Usually DNA synthesis is partially inhibited, but the DNA that is formed appears to be biologically active. This has been shown in phage-infected *E. coli*, since the DNA synthesized in the presence of chloramphenicol can be incorporated into viable phage when the antibiotic is removed or can be involved in genetic

recombination (95). Further, mutations induced in *E. coli* cells in the presence of chloramphenicol can be expressed (47), so that it is possible to conclude that this DNA is genetically functional. It has also been stated (32) that DNA synthesized in human cells in the presence of chloramphenicol is biologically functional. More direct evidence for this idea has now been provided in the work of Goodgal and Melechen (49). These workers have used a transformation assay in *Haemophilus influenzae* to measure the biological functionality of the DNA. The DNA synthesized by *H. influenzae* in the presence of chloramphenicol possesses transforming ability equal to that of normal DNA.

Chloramphenicol-RNA, however, appears to be unstable, since it is degraded and released from the cell under nongrowing conditions, whereas normal RNA remains stable (62). Chloramphenicol-RNA differs from normal RNA in electrophoretic mobility, ultracentrifugal sedimentation rate, and ease of dissociation of its nucleoprotein complex (79). However, its base ratio is normal (62, 79). The instability of this RNA does not always result in its excretion, since in some systems it may be retained within the cell (9).

This alteration in characteristics of RNA is reflected in a change in composition of the ribonucleoprotein particles (ribosomes) of the cell. The ribosomes of growing cells account for 80 to 90 per cent of the RNA of the cell and exist in several sizes with sedimentation constants of 30, 50, 70, and 100 S, of which the 70 S and 100 S components predominate. When chloramphenicol-treated cells are examined, new peaks are found of large concentration at 18 S and 14 S (28). These peaks disappear when the antibiotic is removed, and the normal 29–30 S peak becomes augmented. The 14 S and 18 S peaks differ from normal ribosomes by being very sensitive to sonic oscillation and are disaggregated by Mg^{++} ions but not by citrate. They are composed of 49 per cent RNA and 51 per cent protein, which is more RNA and less protein than in the normal ribosomes. These components are much larger than the soluble RNA, however. Since chloramphenicol inhibits all protein syntheses, it seems reasonable to infer that it inhibits the synthesis of ribosomal protein, and that this inhibition may result secondarily in an alteration in the size of the ribosomes, presumably because there is no new protein being formed to stabilize the newly synthesized RNA. At the same time, the 29–30 S particles break down, and the new smaller 14–18 S particle which is rich in RNA is formed. There is no reason to believe that chloramphenicol has some action specifically restricted to these ribonucleoprotein particles.

Like normal RNA, the RNA synthesized in the presence of chloramphenicol requires a complete supply of amino acids (50). If mutants are used that require various amino acids, RNA synthesis in the presence of chloramphenicol does not occur unless the amino acid is supplied, although only very small amounts of the amino acid are needed and the amino acid supplied is not being incorporated into protein (50). This phenomenon is not observed with every mutant (4), but if the genetic block is not complete, enough amino acid may be synthesized to provide for antibiotic-induced RNA synthesis, or protein degradation (74) may provide enough of the amino acid.

The synthesis of nucleic acids may be inhibited by chloramphenicol under certain specialized conditions. For example, Doudney (33) found with synchronized cultures that DNA synthesis was inhibited when the antibiotic was added just before cell division, whereas RNA synthesis was blocked when the antibiotic was added just after cell division. Presumably enzymes involved in nucleic acid synthesis are formed at certain stages of cell division only, and when the antibiotic is added at that stage, it indirectly prevents nucleic acid synthesis. This sort of explanation is also most likely for the often observed inhibition of DNA synthesis in phage-infected bacteria (27). Since several new enzymes are synthesized in the first few minutes after phage infection which are necessary for the synthesis of phage DNA, if chloramphenicol is added before these enzymes are synthesized, it inhibits DNA synthesis indirectly. An indirect effect of the antibiotic on RNA synthesis in phage-infected cells has also been reported (5). DNA synthesis in mustard-treated (57) and ultraviolet-treated (34) cells is also inhibited by chloramphenicol, presumably because certain new proteins must be synthesized to bring about repair of the damaged DNA before new DNA can be formed.

A discussion of the use of chloramphenicol in

studying the interrelationships of protein and nucleic acid synthesis is given by Hartman and Buchanan (58).

When growing cells are treated with chloramphenicol for 1 to 3 hours, washed, and resuspended in growth medium in the absence of the antibiotic, there is a lag of 30 to 60 minutes before growth resumes (53). By altering various parameters, it has been possible to show that the chloramphenicol lag could be induced only when RNA could be synthesized (13). This lag also occurs if other antibiotics which inhibit protein synthesis are used, such as erythromycin or chlortetracycline, so that it is not specific for chloramphenicol. A similar lag is induced when RNA accumulates during methionine starvation in a particular mutant (11). The significance of this lag is unknown, but it seems related to the accumulation of some abnormal RNA and is not directly related to the mode of action of the antibiotic.

7. Effects on carbohydrate assimilation. The antibiotic does not inhibit carbohydrate assimilation in growing *E. coli* (1). It also does not inhibit the synthesis of an amylopectin-like polysaccharide in resting cells of *Neisseria perflava* (60).

IX. Genetic Studies with Chloramphenicol

The analyses of the processes of mutation and genetic recombination at the molecular level are greatly complicated by the fact that these are usually rare events occurring in an extremely small fraction of the total population. It would be impossible to carry out biochemical analyses which would indicate the processes occurring in the rare mutants or recombinants, so that it is necessary to use indirect procedures. Chloramphenicol has been a useful tool in such studies, since it can be assumed that it is inhibiting protein synthesis in all the cells of the population, even the rare mutants or recombinants.

With the use of this antibiotic it has been possible to examine the involvement or noninvolvement of protein synthesis in these processes.

If cells are irradiated with appropriate doses of ultraviolet light or x-rays and then plated directly on nutrient agar plates, it is found that the number of cells giving rise to colonies varies inversely with the dose of irradiation. If the cells are preincubated on chloramphenicol agar for 1 to 3 hours and then transferred to plain agar, it is found that a large percentage of the cells that did not grow on direct incubation on plain agar do grow after their brief stay on chloramphenicol agar (46). From these results it is inferred that some protein synthesis is necessary before the radiation-induced damage is stabilized.

The mutagenic effects of irradiation are also reversed by chloramphenicol (99). Most of the mutants that would have been expressed after incubation on plain agar are lost after incubation for 1 hour on chloramphenicol agar. This effect is not specific for chloramphenicol, since starvation for nitrogen or essential amino acids, which also inhibits protein synthesis, is just as effective. However, it is operationally simpler to add chloramphenicol, rather than to take away protein precursors.

Genetic recombination in bacteria can occur in several different ways. In bacterial transformation, the uptake of DNA by the cells is not prevented by chloramphenicol (48), although the expression of the transformed character is affected (37). Chloramphenicol does prevent the development of competence (42, 48), presumably by preventing the synthesis of DNA-binding sites in the cell.

Since growth and protein synthesis are not necessary for the transfer of genetic material from cell to cell during mating, it seems probable that chloramphenicol would not prevent this process, although this point has apparently not been investigated.

When cells are infected with temperate phages, either a lytic response or a lysogenic response can occur. Chloramphenicol brings about an increase in the lysogenic response, apparently by inhibiting the protein synthesis that is required for the lytic response (21). Here again the effect is not specific but is brought about also by other conditions that retard biosynthetic operations. There has apparently been no work on the effect of this antibiotic on phage-mediated genetic recombination, but it is probable that this process would also not be affected.

X. Summary and Conclusions

1. Chloramphenicol [D(−) *threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1,3-propanediol] was first discovered as a metabolic product of several *Streptomyces* species.

2. Because of its relatively simple structure,

it was the first clinically useful antibiotic to be synthesized. A large number of analogues of this antibiotic have been synthesized and tested for biological activity, but chloramphenicol itself is the most active compound. A survey of these data indicates that the propane side chain and the hydrogen atoms on carbons 2, 3 and the amide nitrogen are the most likely points of attachment with the critical site in the cell. This *specific* portion of the molecule is modified in its chemical and electronic properties by the less specific *p*-nitrophenyl and dichloroacetyl portions.

3. At low concentrations, chloramphenicol inhibits the growth of a wide variety of bacteria, both gram-negative and gram-positive, as well as rickettsias and certain large viruses. At higher concentrations it inhibits the growth of animal and plant cells. It affects the multiplication of smaller viruses and bacteriophages only indirectly through modification of the host cells. It is inactive against fungi and yeasts. Its activity against bacteria is primarily bacteriostatic, although it may be bactericidal to certain species under certain conditions.

4. Development by bacteria of resistance to this antibiotic is due to mutation and selection, and genetic crosses have shown that high level resistance has a multigenic basis. Cross resistance has been found with the tetracycline antibiotics in enteric bacteria, but not in other species, and this cross resistance has been shown to be due to certain genes that affect the resistance to both antibiotics, in addition to a set of genes conferring resistance to chloramphenicol alone. Nothing is known of the biochemical basis of resistance.

5. Chloramphenicol antagonizes the action of antibiotics which act only on growing cells, such as penicillin and streptomycin, and is additive with other antibiotics which also inhibit protein synthesis, such as the tetracyclines and erythromycin.

6. Although chloramphenicol is weakly antagonized by aromatic amino acids and various other aromatic compounds, there is no evidence that chloramphenicol acts as a competitive inhibitor of some normal metabolite. It is more probable that this antagonism is due to a partial block in the uptake of the antibiotic by the cells.

7. Chloramphenicol does not inhibit oxidative, hydrolytic, or degradative processes, does not prevent the uptake of metabolites into the cell, and does not inhibit the synthesis of small molecules. It also does not inhibit polysaccharide, cell wall, RNA, or DNA syntheses. It does not inhibit the synthesis of simple peptides, such as glutathione and bacitracin. It inhibits total protein synthesis, the synthesis of a wide variety of specific enzymes and other proteins, and the incorporation of radioactive amino acids into protein. It does not inhibit the activation of amino acids or transfer of amino acids to soluble RNA, but prevents some step in their transfer from soluble RNA to protein. Other antibiotics which behave similarly are the tetracyclines, erythromycin, and puromycin.

8. An alteration in the nature and character of bacterial RNA and bacterial ribosomes after chloramphenicol treatment is probably an indirect result of the inhibition of total protein synthesis without inhibition of RNA synthesis.

9. The use of chloramphenicol in variety of genetic investigations is described.

10. Although chloramphenicol is a useful tool in many biochemical studies on macromolecular syntheses, its specific point of action is unknown.

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[3] Because of limitations of space, it has been impossible to cite detailed references to many of the studies discussed. The author will undertake to supply bibliographies on limited areas to workers who write making their needs known.

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